REGENERATION IN THE CARAPACE OF DAPHNIA MAGNA

I. The Relation between the Amount of Regeneration and the Area of the Wound during Single Adult Instars

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In all animals the rates of regeneration appear to have identical characteristics. Przibram (1919) cited the experiments of eleven workers on twenty species and reported that the rates of regeneration are most rapid at the beginning of the process and decrease as regeneration continues. After a study of twelve diverse species, Zeleny (1909*a*) arrived at the conclusion that the rates of regeneration vary directly with the degree of injury up to a maximum, beyond which the rates decrease. Carrel and his colleagues (1916, etc.) found that wound closure in man follows the same general trend, in that the rate of cicatrization is proportional to the area of the wound, but diminishes less rapidly than the area.

The above-mentioned works deal with regeneration of complex structures composed of many tissues and consequently treat with a composite of the rates of individual tissues. A study of the rate in a less complex structure should lead to a better understanding of the fundamental problems involved.

The carapace of *Daphnia magna* is a comparatively simple structure. Two layers of hypodermis (Fig. 1) constitute the cellular tissue of the ventral half of the carapace posterior to the shell gland, the region which is injured by the operative procedure outlined below. Between these layers is a blood chamber and associated with them on their outer surfaces is the chitinous integument. The pillars, which probably also consist of chitin, serve as supporting structures. Figure 2 is a surface view showing the arrangement of the outer hypodermal cells. The markings of the carapace which are characteristic of this cladoceran conform closely to the cell boundaries. The pillars do not appear to be arranged in any definite order.

Sections of the regenerated portions of the carapace are identical with those of the uninjured regions. When viewed from the surface, the shape of the regenerated hypodermal cells is quite variable, in

[1] The greater part of the experimental work upon which this paper is based was carried out at the Zoölogical Laboratory of the State University of Iowa.

contradistinction to that of the original cells. Since the carapace remains the same in cross-section, the difference in the area of the wound from one time to another serves as a three-dimensional measure of the amount of regeneration during the interval.

The purpose of this paper is to present a study of the amount of regeneration in the carapace during single physiological time units, *i.e.* the instars, in relation to the size of the wound under varying conditions

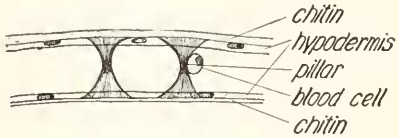

Fig. 1. Camera lucida drawing of a cross-section through the carapace.

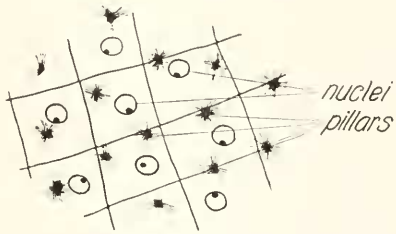

Fig. 2. Camera lucida drawing of the outer surface of the carapace showing the typical arrangement of cells. The surface markings correspond to the cell boundaries. The pillars appear in no definite order.

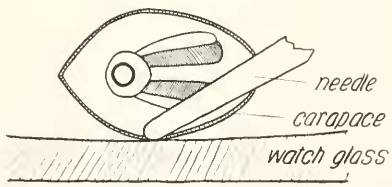

Fig. 3. Diagrammatic section through an animal showing method of operation. Injury to structures other than the carapace is avoided.

of age of the animal, age of the wound, and temperature. These results will be discussed in relation to those secured by others for more complex structures in other forms.

The writer wishes to acknowledge his indebtedness to Dr. L. A. Brown and Dr. J. H. Bodine—to the former for suggesting the problem, and to both for their many helpful suggestions and criticisms as the work progressed.

Materials and Methods

The material, *Daphnia magna* Straus., was secured from Banta's laboratory in 1927. Females from three clones were employed. The original clone was used in the experiments on the effect of age but died out late in 1928. A new clone, started with ephippial eggs from the former in January, 1929, was used in the experiments dealing with temperature effect. Another clone, derived from the latter, was used in the experiments concerned with age of the wound.

Individuals were isolated within twenty-four hours after their re-

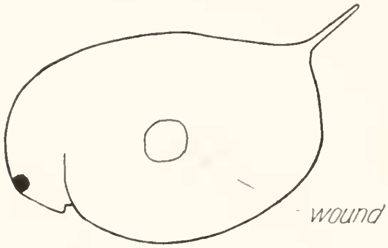

Fig. 4. Outline drawing showing position of wound in carapace.

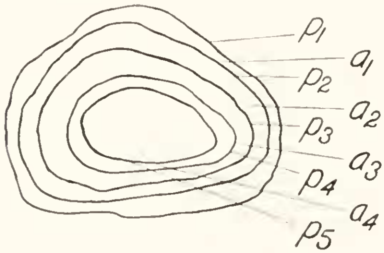

Fig. 5. Camera lucida drawings of a wound during five successive instars. p_1 represents the edge of the wound during the first post-operative instar; p_2, the second; etc. The area a_1 represents the amount of regeneration during the first post-operative instar; a_2, the second, etc. In the graphs which follow, the amount of regeneration during each instar is plotted against the area of the wound during that instar, *i.e.*, a_1 against total area enclosed by p_1; etc.

lease from the brood chamber of the mother and placed in vials containing fresh manure-soil medium (Banta, 1921). The vials used in the experiments on the effect of age and of temperature contained thirty to thirty-five cc. of medium, while those used in the experiments on the effect of the age of the wound contained fifty to sixty cc. of medium. Semi-weekly throughout the experiments one-third of the fluid in each vial was removed and replaced by fresh culture medium.

In the experiments to determine the effect of the temperatures 15° C. and 25° C. the animals were placed, immediately after isolation from the mother, in an electrical refrigerator fitted with a mercury thermoregulator, a heating element, and an electric fan (air temperature ± 0.5° C.). All other experiments were carried on at room temperature (18°–25° C.).

In the operative procedure the animals were placed in watch-glasses and immobilized with a chloretone solution of a concentration just sufficient to bring about cessation of movement. The chloretone did not

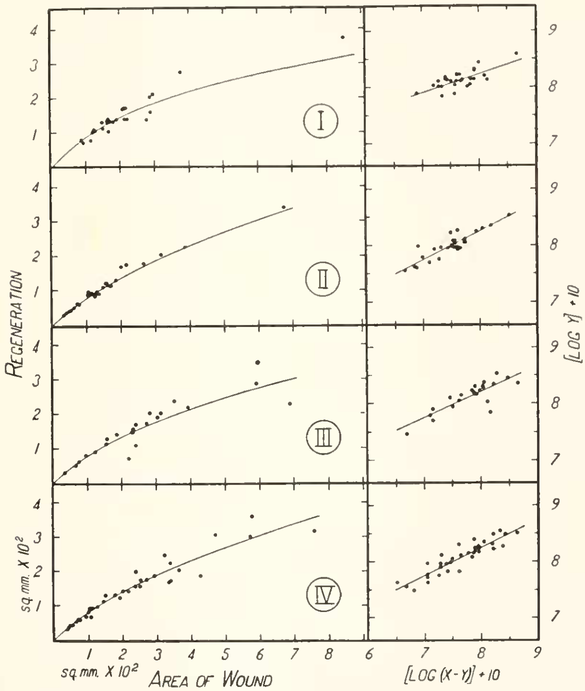

FIG. 6. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instars I–IV. Each animal was operated early in the instar before the one in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

For comparison with later adult instars see Fig. 8.

appear to have any detrimental effects. Part of the area of the carapace in contact with the watch-glass was crushed by applying a steel needle to its inner surface (Fig. 3). Figure 4 shows the position of the wound. The proportions of the wounds were varied by using needle points of different sizes and shapes. Approximately 4000 individuals were operated upon in these experiments.

During the early part of each instar following operation the animals were again immobilized with chloretone and camera lucida drawings (175 ×) made of the wounds. From these drawings the areas were determined by the use of compensating polar planimeters (Keuffel & Esser Co., No. 4242 and No. 4240) and the perimeters by a measuring wheel (Keuffel & Esser Co., No. 1694A) graduated to 1/32 inch.

Experimental

A few hours after an injury is inflicted on the carapace of *Daphnia magna* a brown material forms at the edge of the damaged area. In

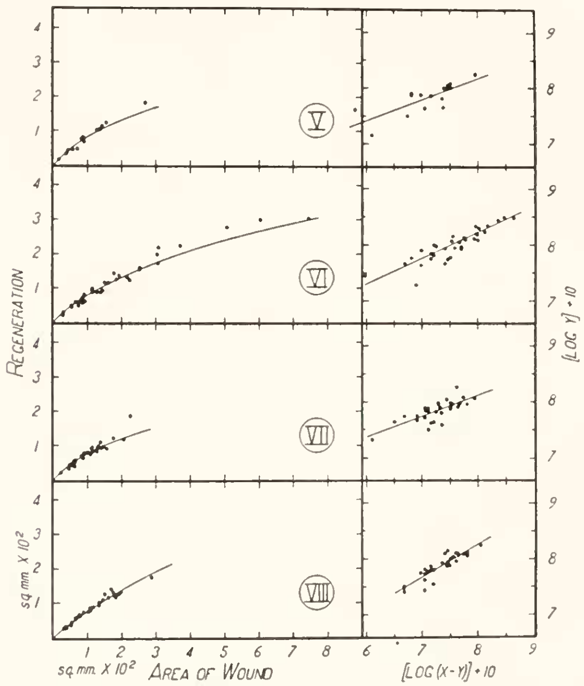

Fig. 7. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instars V–VIII. Each animal was operated early in the instar before the one in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

For comparison with other adult instars see Fig. 8.

all probability this brown material is clotted blood in which the tyrosine has been oxidized (Pinhey, 1930; Anderson, 1932*b*). The brown material is shed with the chitin at the next ecdysis when the wound is made before three-fourths of the instar has passed. However, if the injury is made after three-fourths of the instar has passed, the brown material is retained until the second molt following the injury. If an injury is inflicted early in the instar the edge of the wound during the next instar is smooth and clear. If the injury occurs after half but before

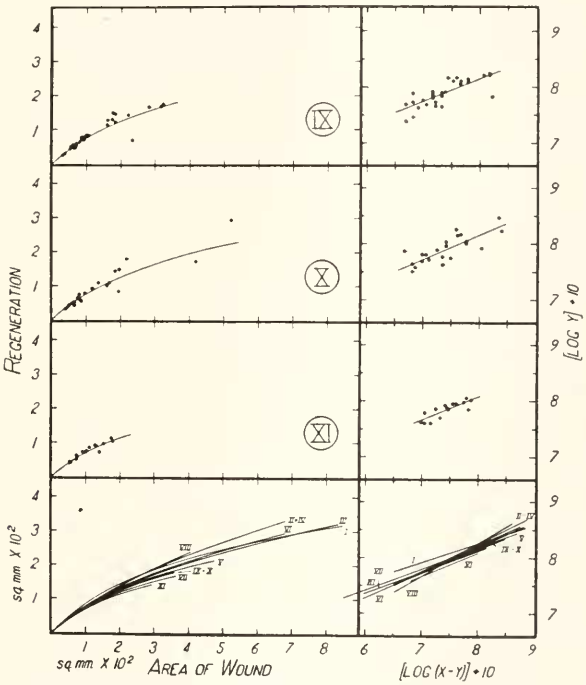

Fig. 8. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instars IX–XI. Each animal was operated early in the instar before the instar in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

The bottom graphs give the curves for each of the adult instars I–XI for purposes of comparison.

three-fourths of the instar has passed, the edge of the wound is rough during the following instar. These phenomena appear to be directly related to the presence or absence of a second layer of chitin at the time of injury (Anderson and Brown, 1930). In all cases, no matter what be the nature of the wound during the first post-operative instar, the edge of the wound is smooth and clear throughout the remaining instars, *i.e.* until complete closure results.

Further, when an operation is performed within the first half of the instar some regeneration takes place before the animal molts. The area of the wound during the following instar is less than that included within the brown ring during the instar of operation. If an operation is performed within the period after half the instar has passed and before three-fourths is over, the wound during the next instar is approximately the same size as the area enclosed by the brown material during the operative instar. In case the injury is produced after three-fourths of the instar has passed, the brown material occupies the same area during the next instar as it does during the instar of injury. Normally wounds diminish noticeably in size by the second post-operative instar irrespective of the time within the instar of operation that the injury is inflicted.

TABLE I

Values of the constants in the equation $y = a(x - y)^k$ expressing the relation between the amount of regeneration (y) and the area of the wound (x) for first post-operative instars with reference to the age of the animals. The data on which these constants are based are shown in Figs. 6, 7, and 8.

Adult instar	Constants		Number of cases
	a	*k*	
I	.09	.35	29
II	.18	.50	29
III	.13	.46	23
IV	.18	.50	35
V	.09	.38	16
VI	.12	.45	34
VII	.07	.37	31
VIII	.27	.59	30
IX	.09	.40	30
X	.10	.43	23
XI	.08	.42	15

The wound remains the same in size and shape throughout any one instar. Change takes place only at ecdysis and shortly thereafter. This is also true for the body length and for the antennal segments of the animal (Anderson, 1930, 1932*a*, *b*) and for the antennæ of *Simocephalus gibbosus* and *Daphnia carinata* (Agar, 1930).

Preliminary experiments showed the impracticability of securing wounds of an exactly identical size and shape. The area injured was always greater than the immediate area crushed. This extended area of injury varied considerably for each case. These experiments also showed that the amount of regeneration during any instar varied with the size and shape of the wound. The shapes may be classified according to the ratios of the perimeter to the square root of the area. The data included in this paper are for wounds where the ratio of the perimeter to the square root of the area does not exceed four. The wounds coming under this classification range from approximate circles to elongated ovals whose lengths rarely exceed twice their widths.

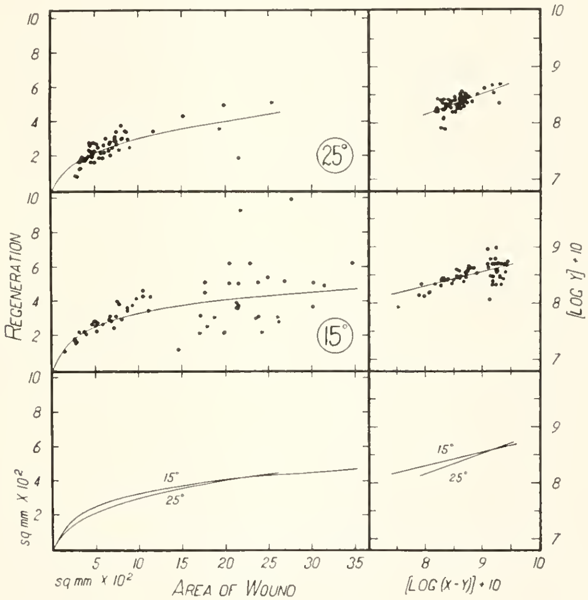

FIG. 9. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instar II at the temperatures 15° and 25° C. Each animal was operated early in the instar before the one in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

With the foregoing facts in mind, a series of experiments was designed to determine the amount of regeneration for wounds of different sizes but of the same approximate shape, controlled by the means outlined above, during each of the first eleven adult instars. In each case the animals were operated early in the instar previous to the one for which records were taken. The results are shown in Figs. 6, 7, and 8. The area of the wound represents size during the instar in

TABLE II

Values of the constants in the equation $y = a(x - y)^k$ expressing the relation between the amount of regeneration (y) and the area of the wound (x) for first post-operative instars with reference to temperature, the age of the animals being the same. The data on which these constants are based are shown in Fig. 9.

Temperature	Constants		Number of cases
	a	k	
° C.			
15	.06	.25	62
25	.08	.36	56

question. The amount of regeneration is the difference between the area of the wound during the instar in question and the area during the following instar (Fig. 5). The results indicate that the amount of regeneration for wounds of identical sizes is approximately the same during the adult instars studied.

Mere graphic presentation of the data yields only a rough means of comparing the results. It was therefore deemed advisable to determine the nature of the relation between the amount of regeneration and the area of the wound. On first glance the relation seems parabolic. The simplest expression for a parabolic curve would be

$$x = ay^k. \tag{1}$$

But the amount of regeneration can never exceed the area of the wound. By adding y to the second member of equation (1) we have

$$x = ay^k + y. \tag{2}$$

By letting y be the amount of regeneration and x be the area, the amount of regeneration can never exceed the area of the wound.

For ease of manipulation, since both x and y are known, equation (2) was converted to

$$y = a(x - y)^k. \tag{3}$$

If this equation is suitable, double logarithmic plots of the amount of regeneration (y) against the area minus the amount of regeneration ($x - y$) should yield a straight line.[2] Such is practically the case as is shown in Figs. 6–11.

At present this equation may be considered only descriptive. No biological significance has been assigned to the constants.

[2] It may be noted that $(x - y)$ is the area of the wound after y has been regenerated.

The constants for each experiment were determined by the method of least squares. The curves in the figures were fitted by means of the constants so determined.

A study of the composite graphs in Fig. 8 and the constants in Table I shows that in general for wounds of the same size the amount of regeneration decreases as the animals grow older.

The amount of regeneration for wounds of identical sizes and shapes is less during the pre-adult instars than during adult instars. The amount of regeneration increases with each instar until the adult condition is reached. The first adult instar is the one during which

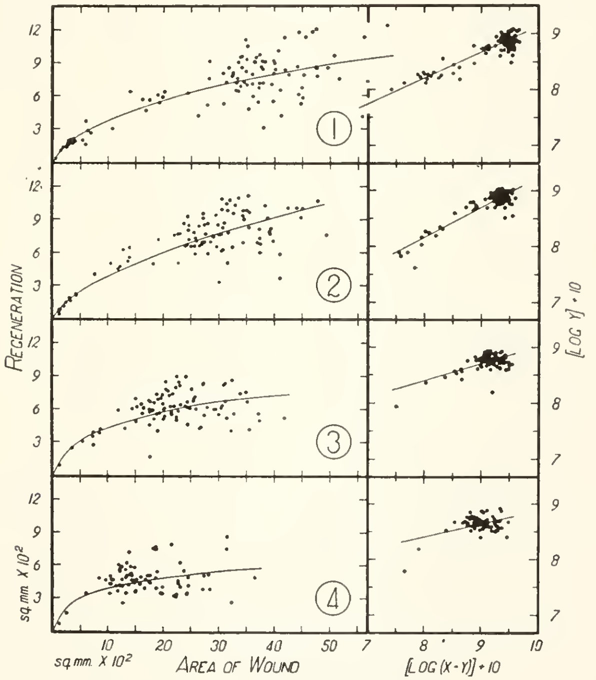

Fig. 10. Graphs showing the relation of the amount of regeneration to the area of the wound for each of the first four instars after operation. Each animal was operated early in the first adult instar. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

For comparison with later instars see Fig. 11.

the first clutch of eggs appears in the brood chamber. Considerable irregularity has been found in the number of pre-adult instars for this species (Anderson, 1930, 1932*a*). Consequently comparisons of the amounts of regeneration for any given pre-adult instar are hardly justifiable at present.

Another series of experiments was carried out to test the effect of temperature on the amount of regeneration during a single instar. Animals which had been kept at specified temperatures (15° and 25°

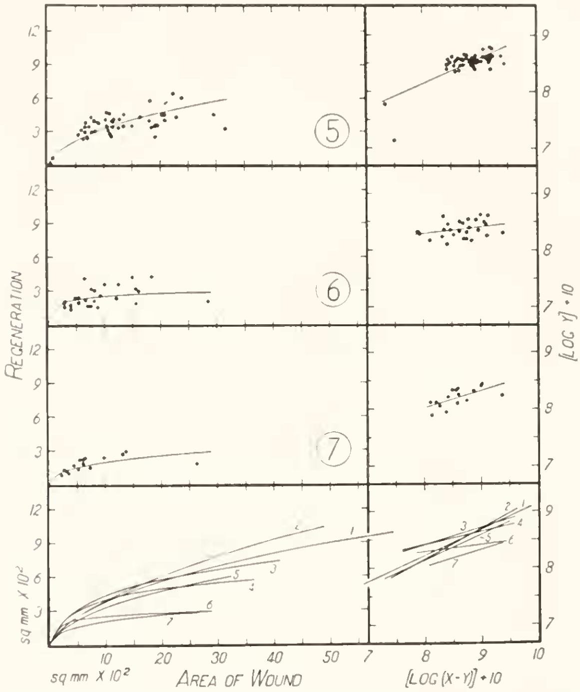

Fig. 11. Graphs showing the relation of the amount of regeneration to the area of the wound for the fifth to the seventh instars after operation. Each animal was operated early in the first adult instar. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

The bottom graphs give the curves for each of the first seven instars following operation.

C.) from the time of their isolation were operated during the early part of the first adult instar. Records were taken for the second adult instar. The results are shown in Fig. 9. The constants are given in Table II. From the figure it may be seen that the difference in temperature within the limits studied has relatively no effect on the amount of regeneration.

The preceding experiments were concerned with relatively small wounds and with the first post-operative instar. Another series of experiments was designed to determine the amount of regeneration for the largest possible wounds and further to determine the effect of the age of the wound on the amount of regeneration. Animals were operated early in the first adult instar and records taken during each successive instar until death ensued. The results are shown in Figs. 10 and 11. The size of the wound is always that during the instar in question as shown in Fig. 5. The results for the eighth and ninth post-operative instars are not shown, since the number of cases was considered too small to be of significance. The values of the constants for each post-operative instar are given in Table III.

Table III

Values of the constants in the equation $y = a(x - y)^k$ expressing the relation between the amount of regeneration (y) and the area of the wound (x) for successive instars following operation. The data on which these constants are based are shown in Figs. 10 and 11.

Post-operative instar	Adult instar	Constants		Number of cases
		a	k	
1	II	.14	.45	105
2	III	.17	.54	102
3	IV	.10	.30	94
4	V	.08	.23	79
5	VI	.11	.45	51
6	VII	.03	.12	28
7	VIII	.04	.30	15
8	IX	.03	.25	10
9	X	.06	.47	5

The results show that the relation tends to be the same for large and for small wounds, during the first post-operative instar. The values of the constants for the first few post-operative instars are almost the same as those where the age of the animal alone was considered. A study of the composite graph in Fig. 11 shows that the amount of regeneration decreases with the increase in the number of instars between the time of operation and the instar during which records were taken and to a greater extent than that where age of the animal alone is considered.

Discussion

Zeleny (1909*a*) pointed out the principal sources of error that arise in quantitative work on regeneration. As he listed them they are: (1)

age, (2) periodic physiological changes, (3) character of the laboratory histories, (4) changes in the rate during the regeneration period, (5) successive regenerations, (6) level of the cut, (7) temperature, (8) food, (9) differences in manipulation, (10) departure of living conditions from the optimum, (11) the relation of the degree of injury to the optimum degree, (12) individual variation. Five of these factors have been made subjects of the present study, namely: age, periodic physiological changes—the instar, changes in rate during the regeneration period, temperature, and relation of the degree of injury to the optimum degree. The other factors have been eliminated in the following manner:

The Character of the Laboratory History.—The animals are well adapted to laboratory conditions since they have been reared in laboratories for well over a decade.

The Level of the Cut.—Zeleny had reference to the amount removed. This factor may be thought of in a somewhat different sense in the present case, for the various regions of the carapace may have different rates of regeneration. The positions of the wounds were as nearly identical as possible in all instances.

Successive Regeneration.—The problem at hand is not concerned with this factor.

Food.—The food used was as uniform as possible. Banta's manure-soil medium was employed in all experiments.

Differences in Manipulation.—As far as possible all individuals in any one series of experiments were subjected to the same manipulations. (See *materials and methods.*)

Departure of Living Conditions from the Optimum.—All animals in any one series of experiments were subjected to the same living conditions. The animals retained their vigor as was evidenced by the continued production of young throughout the experiments.

Individual Variation.—This factor was reduced to a minimum through the use of animals from a single clone in each series of experiments, thus insuring individuals genotypically alike. Large numbers of animals were employed as may be seen from the tables.

Zeleny in his treatment of the above factors suggests that the molting period be used as the center of observations for Crustacea. Indeed, such is the only possible way to handle the above problem. The size and shape of the animals remain constant throughout any one instar. Only at ecdysis and within the period immediately following, during which the chitin hardens, does any normal change take place. The wound remains the same in size as does the carapace of the animal.

Whether or not adult instars are quantitatively equivalent units is a question which remains unsolved. Under optimum conditions females bear a brood of young during each adult instar. At the beginning of each adult instar a number of eggs are passed into the brood chamber. These develop into free-swimming young which are released a few hours before the end of the instar. Usually the time between the release of the young and the end of the instar increases with the age of the animal. The duration of an instar also probably increases with age.

MacArthur and Baillie (1929) and Obreshkove (1930), working with *Daphnia magna* and *Simocephalus expinosus* respectively, found that the metabolic rate decreases with age. If adult instars are quantitatively equivalent units one would expect that the instars would be proportionately longer as the metabolic rate decreases. However, since the amount of regeneration is somewhat less for wounds of the same size during later adult instars than for earlier, one might come to the conclusion that instars are not necessarily equal. Further work on instar length in relation to metabolic rate is necessary to determine the status of the adult instars as quantitatively equivalent units.

Zeleny (1909*a*) found that the rate of regeneration of an organ increases with the degree of injury up to an optimum beyond which the rate decreases. In his experiments several organs were removed and the rate of regeneration for one was compared to the rate when that one only was removed. One must note in the present results that the amount of regeneration for any instar increases with the size of the wound, but the ratio of the amount of regeneration to the area of the wound decreases as the area is increased.

Zeleny (1909*b*) also noted the rate of regeneration for younger animals to be less than for older, but the actual time necessary to replace an organ to be more for older than for younger animals. Du Noüy (1916) showed that the rate of cicatrization in man decreased with the age of the individual. In the case at hand the amount of regeneration during the first post-operative instar for wounds of equal size in adult animals is more in younger than in older animals.

Przibram (1926) has regarded regeneration as an acceleration of normal growth processes. This viewpoint is tenable if the rate of regeneration need not be dependent on the rate of normal growth. Increase in size of *Daphnia magna* females is greatest during the first two adult instars and becomes less with each adult instar up to the tenth, after which time the size of the animals remains relatively constant (L. A. Brown, unpublished data). It may be observed in Fig. 8 that the amount of regeneration decreases with age. The decrease in the amount of regeneration is not equivalent to the decrease in the amount of growth. Godlewski and Latinik (1930) reported that in the tail of the axolotl growth and regeneration are largely independent of each other.

Durbin (1909) found that the rate of regeneration in tadpole tails was quite slow at first but increased to a maximum and decreased to zero after a time. Przibram (1919) concluded from experiments on twenty species from five different phyla that the rates of regeneration are most rapid at the beginning of the process and decrease as regeneration continues. A study of Figs. 10 and 11 shows that the amount of regeneration is greatest at first and decreases as the process continues.

The results here presented agree in general with the findings of Carrel and Hartmann (1916) for the cicatrization of wounds in man, in that the rate is proportional to the area of the wound.

Ebeling (1922) reported that the value of the Q_{10} was 2 for the rate of cicatrization of wounds in alligators. The value of the Q_{10} for the instar length of young adult female *Daphnia magna* is approximately 2.3 between the temperatures 15° and 25° C. (Anderson and Brown, 1930). While instar length varies directly with the temperature, the amount of regeneration is relatively unaffected. The rate of regeneration taken on the basis of absolute time units would therefore be affected by temperature to the same degree as is instar length.

One may conclude that the amount of regeneration during any single adult instar is a parabolic function of the area of the wound during that instar, and is independent of the temperature, but decreases with the age of the animal and to an even greater extent with the age of the wound.

Summary

1. This report deals with a study of the amount of regeneration in the carapace of *Daphnia magna*.

2. The amount of regeneration during any adult instar is a parabolic function of the area of the wound during that instar and may be expressed by the equation

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants.

3. The amount of regeneration during any adult instar decreases with the age of the animal.

4. The amount of regeneration during adult instars is independent of the temperature.

5. The amount of regeneration during any adult instar decreases with the age of the wound but to a greater extent than when the age of the animal alone is considered.

6. These results have been discussed with reference to those secured by others for relatively complex structures.

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